



Genome-wide analysis of gene expression after one year of venom immunotherapy

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ABSTRACT

Background Insect venom immunotherapy (VIT) is used to protect patients against Hymenoptera insects' venom allergy (HVA), which can result in severe systemic or even life-threatening conditions. Molecular mechanisms triggered by VIT remain largely unknown.

Objective To compare genome-wide gene expression of patients with severe HVA prior to VIT and 12 months after.

Methods Whole blood RNA samples were analyzed on an expression array. Results from differential expression obtained on a microarray platform were confirmed by quantitative real-time PCR (qRT-PCR). Subsequently we applied unsupervised clustering. Relative blood cell proportions and gene expression profiles were used as an input to csSAM to compute cell specific differential gene expression. Finally, transcription factor enrichment analysis was performed in MotifLab.

Results & conclusions Comparison of genome-wide expression patterns for whole blood and qRT-PCR experiments revealed no significantly up and/or down regulated genes. This has been corroborated by unsupervised clustering. We found a significant upregulation of 26 genes in macrophages, of 15 genes in monocytes and 2 genes in T regulatory cells (Tregs). Analysis of the promoter sequences of these upregulated genes revealed a significant over-representation of binding motifs specific for kruppel-like factor 4, retinoic acid receptor gamma, and vitamin D receptor.

Our results indicate that changes of gene expression invoked by VIT in peripheral blood may have a too small effect to be detected by conventional biostatistical approaches. When blood cell composition was taken into account we uncovered numerous changes of cell-specific gene expression. Given the regulatory implications we hypothesize that above-mentioned alterations may contribute to activation of anti-inflammatory signals in the innate branch of the immune system.

1. Introduction

Hymenoptera insects venom allergy (HVA) is one of the most serious allergic diseases because of its considerable prevalence and the fact that anaphylactic reactions which may occur in its course can pose a potential death threat. According to various sources, 3–5% of adult population have systemic reactions to Hymenoptera stings [1]. In Poland, a prevalence of 8.9% was found [1,2]. Approximately 10% of these are of anaphylactic nature [3]. The only medical procedure that can protect

HVA patients against such reactions is insect venom immunotherapy (VIT) based on administration of doses of allergens every 4–6 weeks for 3–5 years enables a long-time a long-time (possibly permanent) safety from anaphylactic reactions [4].

Molecular mechanisms triggered by VIT remain largely unknown. The already well recognized immunological markers associated with allergen immunotherapy include: decrease in number and activity of mast cells, basophils and eosinophils, regulation of IgE and IgG an initial rise followed by a decline in allergen-specific IgE correlated with a

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Table 1
Demographic and clinical data.

Patients Number	Age	Sex	Grade of allergic reaction after sting According to Mueller scale	Responsible insect	IDT* with venom of responsible insect - concentration 10 ⁻⁶ g/1 (mm)	slgE** with venom of responsible insect (concentration and class)	Vesp v 1 (concentration and class)	Vesp v 5 (concentration and class)	Api m 1 (concentration and class)	Results of sting challenge / field sting
1	45	F	III	Wasp	12 × 12	12.13 (2)	0.02 (0)	6.82 (3)	0.002 (0)	Negative
2	32	M	IV	Wasp	9 × 11	2.23 (2)	0.03 (0)	10.4 (3)	0.01 (0)	Negative
3	45	M	IV	Wasp	10 × 10	0.589 (1)	0.07 (1)	0.33 (0)	0 (0)	Negative
4	40	M	IV	Wasp	11 × 14	9.15 (3)	11.4 (3)	0.28 (0)	0 (0)	Negative
5	37	M	IV	Wasp	8 × 11	1.86 (2)	ND***	ND	ND	Negative
6	40	F	III	Wasp	10 × 12	7.59 (3)	0.61 (1)	3.47 (2)	0 (0)	Negative
7	50	F	IV	Wasp	10 × 11	5.11 (3)	1.96 (2)	4.88 (3)	0.37 (1)	ND
8	62	M	III	Bee	13 × 16	8.96 (3)	ND	ND	ND	Negative
9	38	F	IV	Wasp	7 × 9	5.2 (3)	0.01 (0)	3.12 (2)	0.004 (0)	ND
10	62	F	IV	Wasp	8 × 10	0.35 (0)	0.003 (0)	1.59 (2)	0 (0)	ND
11	56	F	IV	Wasp	7 × 8	2.15 (2)	ND	ND	ND	Negative
12	71	M	III	Bee	10 × 12	5.61 (3)	0 (0)	0 (0)	0.12 (0)	ND

* IDT - intradermal test.
** slgE – specific IgE.
*** ND - not determined.

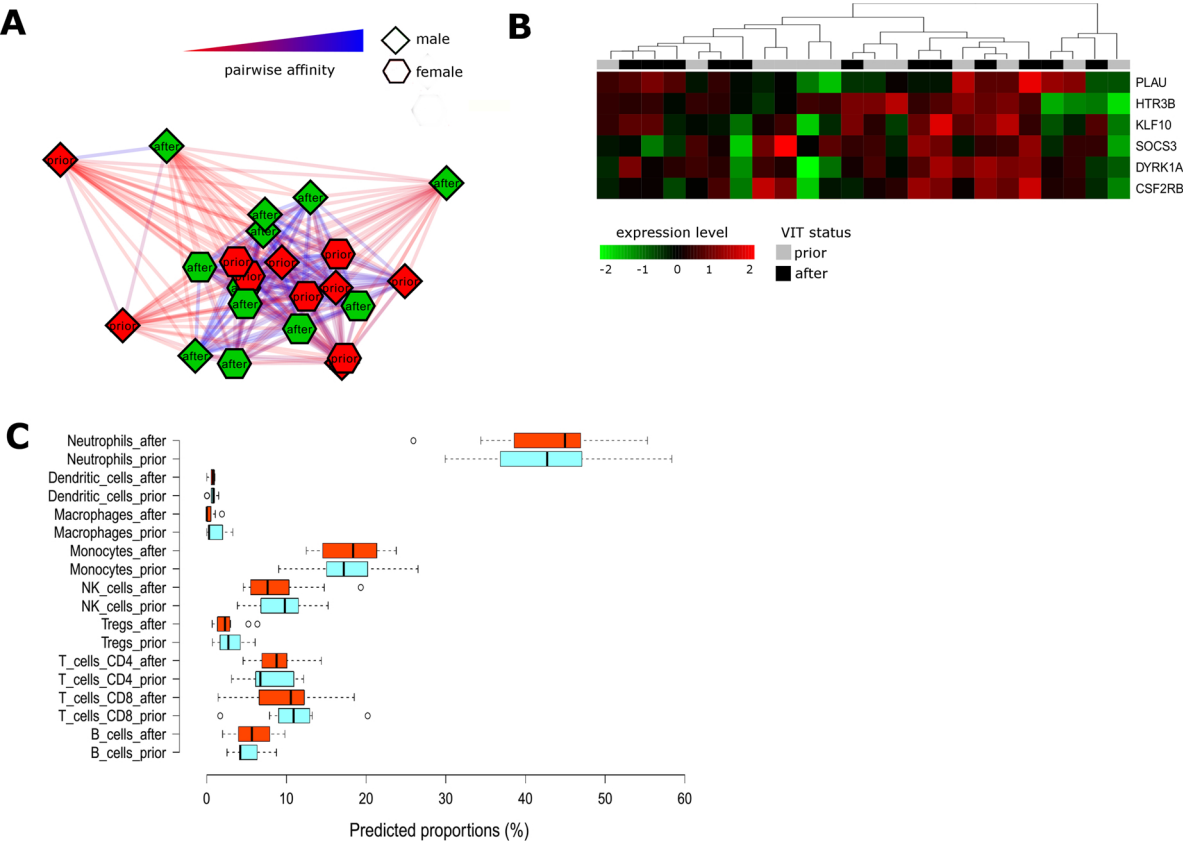


Fig. 1. Bioinformatic analysis of expression array data. (A) Unsupervised, fuzzy AutoSOME clustering visualized in Cytoscape [47]. Node shape and color represents gender and VIT status of the sample, respectively. (B) Clustered heatmap (Euclidean distance, complete linkage) generated based on clustering of $\Delta\Delta Ct$ values of 6 selected genes. For better visualization, rows were scaled to z-score after calculating the dendrogram (C) Box plots representing leukocyte profiles of patients prior and after 1 year of VIT computed from expression data by CIBERSORT algorithm. Note that no statistically significant differences between compared groups were detected.

rise in IgG antibodies, especially in subclasses 1 and 4 and changes in the characteristic of allergen-specific T- and B- responses [5]. More recent studies have indicated a causative relationship between allergen immunotherapy and increase in the frequency of T and B regulatory cells which are important agents in peripheral tolerance [5–7]. In the course of VIT, venom-specific regulatory T (Treg) cells induce allergen

tolerance via suppressing proliferative and cytokine responses to venom allergens. Treg cells affect, directly or indirectly, the response of the effector cells of allergic reaction through cytokine secretion (IL-10 I TGF-beta) and important cell surface suppressive molecules. The second main mechanism that underpins successful immunotherapy is a shift of the T-helper type 2 (Th2) dominated immune response towards a more

Table 2qRT-PCR results of selected genes obtained in a group of 25 patients sampled prior and after 1 year of VIT. $P(H1) \leq 0.05$ was regarded as statistically significant.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)
DYRK1 A	TRG	0,99	0,952	0,627 - 1,453	0,332 - 2,000	0,590
HTR3B	TRG	0,99	1,609	0,266 - 9,936	0,032 - 60,297	0,221
KLF10	TRG	0,99	0,843	0,508 - 1,438	0,328 - 2,146	0,096
SOC3	TRG	0,99	1,033	0,353 - 3,135	0,127 - 9,449	0,879
PLAU	TRG	0,99	0,832	0,326 - 2,071	0,135 - 4,916	0,335
CSF2RB	TRG	0,99	0,835	0,283 - 2,619	0,095 - 10,980	0,458
GUSB	REF	0,99	0,949			
HPRT	REF	0,99	1,053			

Table 3List of genes that were significantly upregulated in blood cell type-specific manner after one year of VIT (in comparison before VIT; FDR ≤ 0.25). Mono - monocytes; macro - macrophages, Tregs - T cells regulatory.

Gene symbol	Gene name	Cell type
<i>BPI</i>	bactericidal/permeability-increasing protein	mono
<i>BTNL8</i>	butyrophilin-like 8	macro
<i>CAMP</i>	cathelicidin antimicrobial peptide	mono / macro
<i>CCR3</i>	chemokine (C-C motif) receptor 3	mono / macro
<i>CEACAM8</i>	carcinoembryonic antigen-related cell adhesion molecule 8	mono / macro
<i>CEBPE</i>	CCAAT/enhancer binding protein (C/EBP), epsilon	mono / macro
<i>CLC</i>	Charcot-Leyden crystal protein	macro
<i>CMTM5</i>	CKLF-like MARVEL transmembrane domain containing 5	macro
<i>DEFA4</i>	defensin, alpha 4, corticostatin	mono / macro
<i>GPR146</i>	G protein-coupled receptor 146	mono / macro
<i>HLA-DQA1</i>	major histocompatibility complex, class II, DQ alpha 1	Tregs
<i>HP</i>	haptoglobin	mono / macro
<i>ITGA2B</i>	integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	macro
<i>ITGB3</i>	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	macro
<i>KEL</i>	Kell blood group, metallo-endopeptidase	macro
<i>LCN2</i>	lipocalin 2	mono / macro
<i>LTF</i>	lactotransferrin	mono / macro
<i>LY6G6F</i>	lymphocyte antigen 6 complex, locus G6F	mono / macro
<i>NINJ2</i>	ninjurin 2	macro
<i>PRSS33</i>	protease, serine, 33	mono / macro
<i>PVRL2</i>	poliovirus receptor-related 2 (herpesvirus entry mediator B)	macro
<i>RNF182</i>	ring finger protein 182	mono / macro
<i>SELP</i>	selectin P (granule membrane protein 140 kDa, antigen CD62)	macro
<i>SLC38A5</i>	solute carrier family 38, member 5	macro
<i>TCN1</i>	transcobalamin I (vitamin B12 binding protein, R binder family)	mono / macro
<i>TNNT1</i>	troponin T type 1 (skeletal, slow)	macro
<i>TREML1</i>	triggering receptor expressed on myeloid cells-like 1	macro
<i>VSTM1</i>	V-set and transmembrane domain containing 1	mono / macro

balanced (Th2/Th1) or Th1-type response. There are also studies suggesting that in patients with IgE-dependent allergy the mechanism of reaction to allergen may involve disturbances in the innate immunity system that might in turn be corrected by allergen immunotherapy [8–10]. Some results indicate that allergen immunotherapy may correct e.g. an impaired function of toll-like receptors (TLRs) on dendritic cells [11]. Thus, the latter studies clearly disclose the complexity of mechanisms involved in allergen immunotherapy.

In searching for molecular basis of a process of acquisition of immune-tolerance during VIT, an analysis of gene expression offers a key method to assess these changes. Up to now, gene expression has been analyzed for immunotherapy to: ovalbumin, grass, house dust mites, and recently also to insect venom [12]. These few studies that focused on studying expression of selected markers in specific fractions of blood

Table 4

List of significant transcription factor binding sites found among 28 significantly upregulated genes. TF- transcription factor.

TF symbol	TF name	Number of genes with given TF binding site (%)	adjusted p-value
KLF4	kruppel-like factor 4	8 (28)	3.8×10^{-9}
RARG	retinoic acid receptor gamma	12 (42)	1.6×10^{-6}
VDR	vitamin D receptor	13 (46)	1.4×10^{-6}

cells in the course of VIT indicated elevated expression of genes coding interleukins (ILT3, ILT4, IL-10), interferon gamma, Foxp3 and specific Th1, Th2 and TCD4+ surface markers in patients subjected to VIT [13,14]. However, to date the number of published studies on genome-wide expression patterns in the context of VIT is limited to three. Konno et al. demonstrated elevated expression of osteopontin (OPN), osteopontin regulatory pathway and cytokine receptors in treated patients when compared to untreated allergic controls [15]. Their results were not confirmed in two subsequent studies by Niedoszytko et al. that analyzed genome-wide expression patterns in the whole-blood of allergic patients in different stages of VIT [16,17]. Niedoszytko et al. found a relatively large number of transcripts that were differentially expressed in patients during or after VIT when compared to patients before VIT. The list of transcripts was significantly enriched for genes involved in cell differentiation, cells interactions, ion transport and signal transduction.

Up to date, no studies were published that monitor whole blood genome-wide expression changes occurring in VIT-treated patients in conditions eliminating potentially confounding factors that influence gene expression, such as interindividual variations in gene expression, patient's state of health as well as seasonal variation in blood white cells composition. Therefore, we compared genome-wide expression in a group of patients with severe HVA who were sampled before starting and after 12 months of VIT.

2. Materials and methods

2.1. Study group

In brief, the group of patients selected for expression microarray consisted of 12: 1/ severe allergic reactions (III°/IV° according to Mueller's classification) after bee or wasp sting, 2/ unquestionable identification of the culprit insect (Patient definitely recognized the particular culprit insect. STs (skin tests) and serum IgE tests confirmed sensitization to the insect pointed at by the patient. There were no positive STs i sIgE test results to any insect other than the one identified by the patient) 3/ venom specific IgE antibodies demonstrated by: positive results of venom-specific intradermal test and serum specific IgE (against whole venom or the major allergen of the venom (Vesp v1, Vesp v2 or Api m1). All patients were subjected to the same treatment protocol (ultrarush). Successful VIT was confirmed in 8 out of 12

patients by a sting challenge test or a field report (Table 1). The real-time PCR (qRT-PCR) experiments were carried out in a larger group of 25 patients that included 12 individuals selected for expression microarray. Blood from each patient was sampled two times (prior and after one year of VIT). Study had been approved by a Wroclaw Medical University ethics committee (internal number 521/2012). All patients gave their written informed consent to participate in the study.

2.2. Expression microarray and real-time PCR

24 whole blood RNA samples were analyzed on expression array (GeneChipPrimeView, Affymetrix). Probe sets were mapped using custom chip definition file (Brainarray, CDF version 20) and normalized with RMA algorithm followed by batch correction (ComBat) [18,19]. Raw data obtained in this study are available from Geo Expression Omnibus: GSE92866. Differential expression was assessed using moderated paired t-test (significance threshold: $FDR \leq 0.05$) implemented in *limma* package [20]. To confirm expression array results we performed quantitative real-time PCR (qRT-PCR) in a group of 25 patients sampled prior and after 1 year of VIT using 6 VIT-related markers (*PLAU*, *CSF2RB*, *KLF10*, *SOCS3*, *HTR3B*, *DYRK1A*). Relative quantification and calculation of the range of confidence was performed using the REST 2009 Software [21].

2.3. Bioinformatics processing of expression microarray data

For unsupervised, fuzzy clustering of array data we used the AutoSOME algorithm [22]. Computation of the relative cell proportions have been performed using the CIBERSORT software [23]. Cell proportions were compared between groups using the Kruskal-Wallis test implemented in *compare Groups* package [24]. Relative cell proportions predicted by the CIBERSORT and gene expression profile were used as an input to *csSAM* to compute cell specific differential gene expression (significance threshold: $FDR \leq 0.05$; fold change ≥ 1.5) [25]. Transcription factor enrichment analyses were performed in MotifLab software [26].

3. Results

3.1. Supervised and unsupervised analysis of genome-wide gene expression profiles

In this study we conducted prospective genome-wide expression analysis of approximately 19000 transcripts in a group of 12 patients prior and after 1 year of VIT (Table 1). First we applied supervised analysis of differences between studied groups of expression profiles measured in the whole blood white cells that revealed no significantly up and/or down regulated genes between studied subgroups (data not shown).

We performed unsupervised, fuzzy clustering to visualize mutual relations between the samples. As shown in Fig. 1A a pairing between majority of patients reveals strong similarity between their expression patterns regardless of the gender or VIT status.

Next, we used qRT-PCR to compare the expression of 6 genes that had been reported by Konno et al. (*PLAU*, *CSF2RB*) and Niedoszytko et al. (*KLF10*, *SOCS3*, *HTR3B*, *DYRK1A*) who demonstrated in their studies a significant differential expression of these genes in patients during the maintenance phase of VIT [15,17]. None of selected genes revealed significant alteration when comparing our study groups (Table 2). As shown in Fig. 1B a hierarchical clustering of $\Delta\Delta Ct$ values of 6 selected genes reveals lack of separation of patients according to VIT status.

3.2. Analyses of cell-type-specific expression profiles and transcription factor motifs occurrences

Since during the analysis of whole blood white cells, cell-type heterogeneity may mask biological differences among studied cases we applied a deconvolution algorithm (CIBERSORT) to provide estimates of the relative proportions of several blood cell subtypes (see Fig. 1C). When blood cell relative proportions were taken into account differential expression analysis uncovered significant upregulation of 26 genes in macrophages, 15 genes in monocytes and 2 genes in T regulatory cells (significance threshold (FDR) ≤ 0.05 ; Table 3). None of the studied gene transcripts displayed downregulation in this comparison. The main function of the upregulated genes was stimulation of the primary immune response by affecting: class I MHC mediated antigen processing and presentation (*RNF182*), intracellular signaling (*SELP*), cell chemotaxis and immune mediator induction (*CAMP*), signal transduction (*LCN2*) and cell-cell signaling (*CMTM5*, *ITGA28*; *ITG2B*). Moreover, we also observed upregulation of genes influencing activity of cells participating in known immunotherapy mechanisms such as genes related to production of T lymphocyte cytokines (e.g. *BTN8*; *CCR3*) as well as to accumulation and activation of eosinophils and basophils (e.g. *CCR3*, *CLC*) [5,27,28].

Thereafter, we counted the number of times each transcription factor motif occurred in the upstream promoter sequence of significantly differentially expressed genes and used the expected motif frequencies to calculate p-values for overrepresentation. The following 3 motifs were found to be overrepresented at a significance threshold of 0.05: kruppel-like factor 4 (*KLF4*), retinoic acid receptor gamma (*RARG*) and vitamin D receptor (*VDR*) (Table 4).

4. Discussion

Our comparison of expression profiles of patients prior and after one year of VIT measured in whole blood (regardless of blood cell composition) revealed that expression changes invoked by VIT during one year are likely to have a too small effect to be detected by supervised and unsupervised methods when the cell type composition of the samples is not taken into account. Overall, our results contradict previously reported findings in the context of VIT that suggested a number of significantly deregulated gene targets. Niedoszytko et al. and Kono et al. identified a relatively large number of gene transcripts differentially expressed in patients during the maintenance phase of VIT (3–5 years) by comparison of two different groups of patients, one consisting of patients 3–5 years after VIT and another consisting of patients prior to being treated with VIT [15–17]. There are several reasons that might explain the discrepancies between our and the previous studies. Our study design is unique in this context as we studied gene expression prospectively in patients whose blood samples were collected before and after 12 months of successful venom immunotherapy. It should be also underlined, that our prospective design is less bias-prone than other studies, as a careful attention has been paid to avoid such confounding factors as: batch effects, inter-individual variation in gene expression (by analyzing prospectively identical patients), variation related to ageing and inter-seasonal variation in gene expression (as blood collection before and after VIT have been performed during the same season) [29,30]. Finally, the molecular mechanisms of venom tolerance studied by us in a shorter phase of VIT (12 months of treatment) may be different from those that determine long term tolerance (> 3 years of treatment).

To overcome limitations imposed by use of highly heterogeneous tissue such as whole blood we decided to employ a gene expression deconvolution approach that enables to compute relative blood cell proportions in each sample and facilitates calculation of cell type-specific gene expression differences between patient groups [25,31]. This allowed us to identify a number of genes that were differentially expressed in a cell specific manner in small cell subpopulations present in

whole-blood-white-cells without employing sorting procedures. The latter may result in an introduction of artifacts into a gene-expression patterns [32,33]. As shown by our analysis of cell-specific differential expression the changes mainly concerned upregulation of leukocyte-specific genes expressed in an antigen-presenting cells: macrophages and monocytes.

Remarkably, transcription factors binding sites (KLF4, RARG, VDR), overrepresented in a significant proportion of upregulated genes, indicate an involvement of key immune modulatory pathways in VIT. In particular, recent reports revealed that KLF4 is involved in inflammatory monocyte differentiation, macrophage M1/M2 polarization and activation [34,35]. Interestingly, it has been shown that KLF4 induces anti-inflammatory IL-10 expression in various tissues and cells including macrophages [36]. Retinoic acid (active metabolite of vitamin A) together with its receptors (RARs) are important modulators that may increase immune suppression and anti-inflammatory signals [37]. For example it has been demonstrated that retinoic acid attenuates airway inflammation by inhibiting Th2 and Th17 differentiation and/or functions [38]. VDR is widely expressed on the surface of various immune system cells including T cells, B cells, macrophages, dendritic cells, and epithelial cells [39]. Along with vitamin D supplementation VDR has been shown to be associated with immune system suppression [40]. Importantly, recent pilot studies on allergen immunotherapy have provided the evidence that vitamin D supplementation may improve its effectiveness [41,42].

Taken together, our results support known immunological observations indicating the relevance of monocytes, macrophages and mechanisms of innate immunity in achieving VIT effectiveness after one year. Magnan et al. reported that VIT induces monocyte activation characterized by overproduction of IL-12 and TNF- α [43]. Moreover, they postulated that VIT-induced tolerance could depend not only on specific action of the T cell venom antigens but also on a secondary, non-specific functions of monocytes [43]. Recently, Tversky et al. noted that dendritic cells obtained from allergic patients induced an impairment of IFN- α production following toll-like receptor 9 (TLR-9)-dependent innate immune stimulation [11]. They found that subcutaneous allergen immunotherapy (SCIT) increases dendritic cells TLR-9 mediated innate immune function which was previously impaired. Other studies documented changes in the phenotype of blood monocytes after venom immunotherapy characterized by an induction of molecules such as ILT-3, ILT-4 and secretion of IL-10 [9]. Lastly, alterations in expression of function-associated surface molecules of DCs were also described in the course of immunotherapy in patients allergic to food or house dust mites [44,45]. However, none of these studies established any firm link between changes in blood monocyte function and clinical effectiveness of immunotherapy. Thus, on the basis of our gene expression study we are inclined to hypothesize that while mitigation of T lymphocytes effects is considered to be an important mechanism in achieving venom antigen immunoprotection, a simultaneous restoration of a complementary immune system innate branch also plays a key role in this process.

Potential limitation of our study is the fact that we used whole blood as the mRNA source, whereas significant changes of gene expression in VIT are very likely limited to a small subset of cells. In this scenario presence of an overwhelming proportion of cells not involved in VIT (e.g. neutrophils) may weaken cell subset-specific information [31]. Therefore, analysis of gene expression in low-frequency types of cells by combining genome-wide expression in PBMCs and deconvolution-based cell-specific gene expression seems to be a reliable and less bias-prone procedure [23,46].

4.1. Conclusions

In summary, immune-tolerance is mediated by specific types of nucleated cells, representing a small pool among all whole blood white cells. Identifying alterations of gene expression patterns in these cells is

a challenge and may remain impossible without accounting for the cell-type composition. Our findings uncovered cell-specific gene expression changes induced at the relatively early phase of VIT (1 year) that are related to activation of anti-inflammatory signals in the innate branch of the immune system, which has been recently accepted as key mechanism in building of immune tolerance.

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Conflicts of interest

None to declare

Data availability

Raw data obtained in this study are available from Geo Expression Omnibus: GSE92866

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